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Splice Variants of the α Subunit of the G Protein G_s Activate Both Adenylyl Cyclase and Calcium Channels

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Signal transducing guanine nucleotide binding (G) proteins are heterotrimers with different α subunits that confer specificity for interactions with receptors and effectors. Eight to ten such G proteins couple a large number of receptors for hormones and neurotransmitters to at least eight different effectors. Although one G protein can interact with several receptors, a given G protein was thought to interact with but one effector. The recent finding that voltage-gated calcium channels are stimulated by purified G_s , which stimulates adenylyl cyclase, challenged this concept. However, purified G_s may have four distinct α -subunit polypeptides, produced by alternative splicing of messenger RNA. By using recombinant DNA techniques, three of the splice variants were synthesized in *Escherichia coli* and each variant was shown to stimulate both adenylyl cyclase and calcium channels. Thus, a single G protein α subunit may regulate more than one effector function.

PURIFIED PREPARATIONS OF G_s , the stimulatory regulatory component of adenylyl cyclase, or α_s , the α subunit of G_s , modulate cardiac and skeletal muscle T-tubule Ca^{2+} channels in the absence of cytoplasmic second messengers (1). This finding was surprising for previously there was little indication that a single G protein regulated more than one effector (2), and the possibility existed that the effect might have been due to a contaminating G protein. To determine whether G_s did indeed regulate more than one effector and whether G proteins are potential branchpoints for information transfer across membranes, we tested whether α_s subunits that were synthesized by recombinant methods and that are known to stimulate adenylyl cyclase (3) would also act on Ca^{2+} channels.

G proteins are $\alpha\beta\gamma$ heterotrimers that share a common set of $\beta\gamma$ dimers and differ in the composition of their α subunits. The α subunits are similar yet distinct gene prod-

ucts that bind and hydrolyze guanosine triphosphate (GTP) and act on specific effectors. Because of their similarities, G proteins are often isolated together, and what appears to be a single G protein on several electrophoretic systems may in fact represent more than one. Furthermore, in the case of α_s , as many as four closely related splice variants may be expressed in a single cell (4). The α_s splice variants comprise a pair of shorter and a pair of longer molecules, α_s -S and α_s -L, which differ by a block of 15 amino acid residues that are encoded by exon 3 of the natural gene. Each pair in turn is formed by two polypeptides that differ by the absence (α_s -S1 and α_s -L1) or presence (α_s -S2 and α_s -L2) of a single serine residue at the insertion-deletion junction (4). The relative proportions in which the variants with and without serine are represented in purified G_s is unknown because they cannot be separated. Thus, we examined whether a single α_s polypeptide can activate both adenylyl cyclase and Ca^{2+} channels, and whether this property is common to all of the α_s splice variants.

Previous studies have shown that it is possible to synthesize in *Escherichia coli* biologically active α_s polypeptides as judged by their ability to activate adenylyl cyclase (3). To determine whether the same molecular form of α_s that stimulates adenylyl cyclase also stimulates Ca^{2+} channels, we synthesized in *E. coli* three of the four splice

variants cloned previously (4, 5), using two of the T7 promoter-based expression systems of Tabor and Richardson (6), pT7-5 (3) and pT7-7 (7). For some experiments the cDNAs that encode α_s -S1 and α_s -L1 were subcloned into the Bam HI site of the cloning cassette of the pT7-7 vector. This led to the expression of fusion polypeptides with an amino-terminal extension of nine amino acid residues, termed $r(+9)\alpha_s$ -S1 and $r(+9)\alpha_s$ -L1. For other experiments, the cDNAs that encode α_s -S2 and α_s -L1, together with an appropriate ribosomal binding sequence, were inserted into plasmid pT7-5 so that $r\alpha_s$ -S2 and $r\alpha_s$ -L1 were expressed with an unaltered amino acid sequence (3). For control, the cDNA of the α subunit of another G protein, which does not stimulate adenylyl cyclase, human liver G_i -3 (8), was also subcloned into pT7-7 so as to direct the expression of the corresponding $r(+9)\alpha_i$ -3 (7). Two methods were used to induce expression of the recombinant proteins (7): infection with a phage containing the T7 RNA polymerase gene controlled by the *lac* promoter (plasmid-phage method) or transfection with a second plasmid that carries the T7 RNA polymerase gene under the control of the leftward λ promoter and the temperature-sensitive cI857 repressor (two-plasmid method).

When fusion proteins encoded in pT7-7 constructs were expressed by the plasmid-phage method (7), the recombinant polypeptides accumulated within 60 min of induction and constituted about 5 to 8% of total bacterial cell protein. However, only 5 to 10% of the recombinant α subunits could be recovered as soluble proteins after bacterial lysis with lysozyme and centrifugation (7). When the pT7-5 constructs were used (3), total expression of the recombinant polypeptides was less (about 0.5% of total cell protein), but about 50% was soluble. Thus, the final yield of activity was similar for pT7-7 and pT7-5 constructs.

Two series of experiments were performed to evaluate the effects of recombinant G protein α subunits on Ca^{2+} channel currents produced by skeletal muscle T-tubule vesicles incorporated into planar lipid bilayers (9). In the first, $r(+9)\alpha_s$ -S1 and $r(+9)\alpha_s$ -L1 were expressed by either the plasmid-phage or the two-plasmid method, recovered in lysates, activated with guanosine-5'-O-(3-thio)triphosphate (GTP γ S), and tested either directly or after partial purification over DEAE Sephadex (7). In the latter case, about 20% of the applied $r(+9)\alpha_s$ was recovered with an apparent purity of about 5%, if derived from bacteria induced by the plasmid-phage method, and less if derived from bacteria induced by the two-plasmid system. In the second series of experiments, $r\alpha_s$ -S2 and $r\alpha_s$ -

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L1 (nonfusion polypeptides) were expressed by the two-plasmid method and purified to homogeneity (3). Their effects on Ca^{2+} currents were compared with those of $r(+9)\alpha_s$ -S1, $r(+9)\alpha_s$ -L1, and purified human erythrocyte (hRBC) G_s . As documented elsewhere (3, 7), all forms of recombinant α_s are capable of activating adenylyl cyclase.

All measurements of Ca^{2+} channel currents were carried out in the presence of the dihydropyridine Ca^{2+} channel agonist Bay K 8644. In each test, single-channel currents were compared for 5 to 10 min immediately before and after addition of test fractions. Single-channel Ca^{2+} currents occurred stochastically in bursts and clusters of bursts whose details were obscured by the limited bandwidth of the planar lipid bilayer (Fig. 1). These currents were averaged over 2- to 4-min periods and gave constant values when computed over periods of 20 to 30 min, provided the cis chamber was washed free of vesicles after their incorporation (1). We found that addition of partially purified GTP γ S-activated $r(+9)\alpha_s$ -S1 to the cis chamber produced a clear increase in current (10). In all cases the increase in current was due to an increase in the opening probability of the channels; the slope conductance of the majority (about 90%) of channels was

10 pS between -10 and 10 mV and was unchanged by the stimulatory $r(+9)\alpha_s$ (Fig. 1). The effect was asymmetrical and specific for α_s . Addition of $r(+9)\alpha_s$ to the trans chamber produced no response and $r(+9)\alpha_i$ -3 added to either chamber had no effect (Figs. 1 and 2). In other experiments $r(+9)\alpha_s$ -S1 was effective in stimulating cardiac single-channel Ca^{2+} currents, observed after incorporation of bovine cardiac sarcolemmal vesicles into phospholipid bilayers (11). These experiments prove that a single splice variant of α_s , $r(+9)\alpha_s$ -S1, is capable of activating both Ca^{2+} channels and adenylyl cyclase. Moreover, the effects on Ca^{2+} channels are the same as those shown previously for G_s purified from hRBC, endogenous G_s coinorporated with skeletal muscle T-tubule Ca^{2+} channels, and endogenous G_s coinorporated with cardiac muscle Ca^{2+} channels (1).

We next examined the ability of other splice variants of α_s to activate Ca^{2+} channels using homogeneous preparations of α_s -S2 and α_s -L1 (nonfusion proteins) as well as a partially purified preparation of $r(+9)\alpha_s$ -L1. All of these preparations gave results that were qualitatively and quantitatively similar to each other or to those described above (Fig. 2). Thus, three of the

four known splice variants of α_s could activate both Ca^{2+} channels and adenylyl cyclase, leading to the conclusion that regulation of the specificity of effector interactions is not provided by multiple forms of α_s polypeptides and that contamination of native G_s was not a factor in our earlier experiments (1).

The interactions of $r\alpha_s$ -S2 and $r\alpha_s$ -L1 with adenylyl cyclase and other components of this signal transduction pathway have been studied (3). These recombinant polypeptides appear to interact normally with guanine nucleotides, receptors, and G pro-

Fig. 1. Activation of skeletal muscle T-tubule Ca^{2+} channels by the recombinant short 379 amino acid α_s fusion polypeptide [$r(+9)\alpha_s$ -S1] but not by the recombinant α_i -3 fusion polypeptide [$r(+9)\alpha_i$ -3]. Ca^{2+} channels from skeletal muscle T tubules were incorporated into planar lipid bilayers and single-channel trans to cis currents were measured at a holding potential of 0 mV using Ba^{2+} as the charge carrier (9). Baseline currents were recorded for 5 to 10 min. Preparations of $r(+9)\alpha_s$ -S1 and $r(+9)\alpha_i$ -3, activated by treatment with GTP γ S, purified partially by DEAE-Sephacel chromatography (7), and concentrated by ultrafiltration (12), or control (Con) solutions, were added as 10- to 20- μ l aliquots to the 0.2-ml cis chamber and recording was continued for an additional 5 to 10 min or until the bilayer broke down. (A) Single-channel Ca^{2+} currents displayed at two time resolutions were recorded during a control period before addition of recombinant α subunits, after the addition of 4 nM partially purified GTP γ S-activated recombinant α_i -3 protein prepared by the plasmid-phage method [$r(+9)\alpha_i$ -3], and after addition of 2.4 nM partially purified GTP γ S-activated $r(+9)\alpha_s$ -S1 protein (28 pM G_s equivalents of cyc^- reconstituting activity). (B) Average currents from incorporated Ca^{2+} channels before and after addition of 4 nM GTP γ S-activated $r(+9)\alpha_s$ -S1 protein (equivalent G_s activity = 34 pM) prepared by the two-plasmid method. The probability of an opening event, P , produced by any one of N incorporated channels was calculated from records such as those shown in (A) to give NP values. If one assumes the unitary amplitude to be constant, which is reasonable because the 10-pS conductance accounts for about 90% of all openings (1), NP represents the current. NP was integrated every 0.5 s to produce the experimental diary shown. (C) Cumulative NP values computed from the experiment in (B). The effect of $r(+9)\alpha_s$ -S1 shown is representative of more than 20 other such experiments.

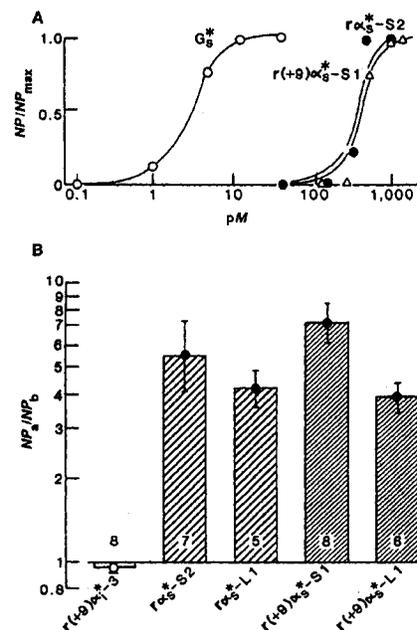
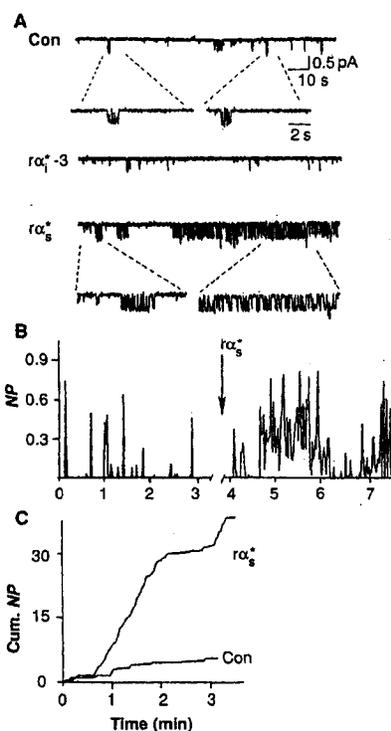


Fig. 2. Stimulation of Ca^{2+} channel activity by individual recombinant α_s splice variants. (A) Concentration-effect curves for stimulation of single-channel Ca^{2+} currents by GTP γ S-activated forms of G_s and two recombinant splice variants. hRBC G_s ; GTP γ S-activated native G_s from human erythrocytes; $r\alpha_s$ -S2, purified; GTP γ S-activated recombinant 380-amino acid α_s prepared by the two-plasmid method; $r(+9)\alpha_s$ -S1, partially purified GTP γ S-activated recombinant 379-amino acid α_s with 9-amino acid amino-terminal extension, prepared by the plasmid-phage method. Each point is the average of two measurements, and the points were normalized to the maximum value. (B) Relative stimulations of incorporated Ca^{2+} channels by recombinant splice variants expressed as ratios of NP after addition of the recombinant GTP γ S-activated polypeptides to NP before their addition (NP after/ NP before). Values are geometric means \pm SEM of the number of experiments shown. The concentrations of recombinant subunits used varied between 0.6 and 4 nM of recombinant polypeptides ($r\alpha_s$ -S2, $r\alpha_s$ -L1) or 2 to 10 pM equivalents of human erythrocyte G_s activity [$r(+9)\alpha_s$ -S1 and $r(+9)\alpha_s$ -L1]. GTP γ S-activated $r(+9)\alpha_i$ -3 [$r(+9)\alpha_i$ -3] at 0.5 to 4 nM, added in some experiments before addition of the recombinant α_s subunits, was always without effect.

tein $\beta\gamma$ complexes. Although they can activate adenylyl cyclase maximally (compared to liver G_s), they appear to have an affinity for the enzyme that is reduced to approximately one-tenth when tested on soluble adenylyl cyclase and to approximately 1/50 when tested on adenylyl cyclase in intact membranes (3). We have suggested that this defect is due to a failure of the recombinant α subunit to undergo a posttranslational modification that normally occurs in eukaryotic cells (3, 12). The potencies of $r(+9)\alpha_s$ -S1 and of α_s -S2 relative to that of a native α_s for stimulating Ca^{2+} channels were therefore estimated. This was done by measuring changes in average current produced by several concentrations of either subunit. The measurements were made between 5 and 10 min after addition of GTP γ S-activated G_s protein or recombinant α_s subunits. In most experiments, however, only one or two concentrations were tested on each planar bilayer, and these were concentrations expected to produce greater than 100% increases in currents. For hRBC GTP γ S-activated G_s (hRBC G_s^*) threshold concentrations were about 1 pM and maximal effects were obtained with 10 to 50 pM hRBC G_s^* (Fig. 2A). This agrees with the 1 to 2 pM required for doubling the activity of adenylyl cyclase from *cyc*⁻ cells, the bioassay that defines G_s activity. Supermaximal stimulation of Ca^{2+} currents by hRBC G_s^* was obtained at 50 to 100 pM [$n = 8$ (1)]. Thus, native hRBC G_s^* has roughly the same potency for activation of Ca^{2+} channels and adenylyl cyclase. For $r(+9)\alpha_s^*$ -S1, prepared by the plasmid-phage method, threshold effects on Ca^{2+} channel activity were obtained at about 200 pM of the recombinant protein, which corresponds to about 2 pM of hRBC G_s^* activity equivalents. Essentially the same result was obtained with the pure, nonfusion α_s -S2 polypeptide (Fig. 2). The two classes of recombinant subunits, α_s -S2 and $r(+9)\alpha_s$ -S1, gave maximal effects at 1 to 4 nM (13) (Fig. 2), which corresponds to 40 to 80 pM G_s activity equivalents.

Additional experiments were carried out with fusion proteins prepared from bacteria induced with the two-plasmid method in which the exact chemical concentration of $r(+9)\alpha_s$ -S1 was not determined, but in which the *cyc*⁻ reconstituting activity in terms of native hRBC G_s^* activity was measured reliably. With these preparations stimulation of Ca^{2+} channel activity to values greater than two times control was obtained at 0.5 to 3 pM of hRBC G_s^* activity equivalents ($n = 7$). Thus, α_s -S2 and $r(+9)\alpha_s$ -S1 have the same relative ability to stimulate both Ca^{2+} channels and adenylyl cyclase. These data suggest that the interactions of α_s with both Ca^{2+} channels and adenylyl

cyclase may involve similar domains of the G protein α subunit. The fact that the potency of α_s -S2 and $r(+9)\alpha_s$ -S1 is very similar is of interest for it indicates that the amino-terminal extension on the α subunit fusion protein does not prevent interactions with effector molecules.

Our results indicate that a particular G protein can serve as a branch point in pathways of transmembrane signaling and suggest that the networks "wired" in the plasma membrane may be quite complex. Maguire and co-workers (14) have demonstrated that the ability of β -adrenergic agonists to inhibit Mg^{2+} influx in S49 cells depends on both receptors and G_s but appears to be independent of adenosine 3',5'-monophosphate (cAMP) and, presumably, adenylyl cyclase. Thus, interactions of G_s with effectors may not be limited to those with dihydropyridine-sensitive Ca^{2+} channels and adenylyl cyclase.

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9. Rabbit skeletal muscle transverse tubule membranes (T tubules) were prepared according to M. Roseblatt, C. Hidalgo, C. Vergara, and I. Ikemoto [*J. Biol. Chem.* **256**, 8140 (1981)], with minor modifications, and stored at -80°C until used. The membrane fractions recovered from the 10 to 20% sucrose interface had specific dihydropyridine binding activities, assayed at 10 nM [^3H]-labeled PN200-110, of 10 to 20 pmol per milligram of protein, and were stored until used at -70°C in 0.3M sucrose at 20 to 30 mg of protein per milliliter. Lipid bilayers with capacitances between 100 and 400 pF were painted over a 100- μm -diameter hole in a Lexan partition separating two chambers of 0.2-ml volume. The bilayers were made of a 20 mg/ml solution containing equimolar concentrations of brain phosphatidylethanolamine and phosphatidylserine in decane (Avanti Polar Lipids, Birmingham, AL). For incorporation of skeletal muscle T-tubule vesicles, the trans chamber was filled with 50 mM NaCl, 2 mM $MgCl_2$, 3 μM Bay K 8644, and 5 mM Hepes adjusted to pH 7.0 with tris base (solution A), and the cis chamber, to which the membrane vesicles were added, received solution A plus 100

mM $BaCl_2$ (solution B). Skeletal muscle T-tubule membrane vesicles are inside-out with respect to the orientation of their cytoplasmic face and incorporate into bilayers exposing their extracellular face to the trans chamber. After incorporation both chambers were washed and the composition of the solutions was exchanged so that the cis chamber contained solution A and the trans chamber contained solution B. This removed membrane vesicles, preventing further incorporation during the course of the experiment, and reduced possible interference of high concentrations of Ba^{2+} with G protein stability or G protein regulation of ion-channel activity. Inward (trans to cis) Ba^{2+} currents gave downward deflections. The currents were filtered at 50 to 100 Hz with a four-pole Bessel filter and digitized at rates that were at least four times the Nyquist frequency. Single-channel currents were detected by a half-amplitude detection method [J. Colquhoun and F. J. Sigworth, in *Single Channel Recording*, B. Sakmann and E. Neher, Eds. (Plenum, New York, 1983), pp. 191-263] and were converted to idealized events in which transitions from closed to open and vice versa were treated as instantaneous. Further analysis was done with interactive programs and a PDP-11/73 computer [H. D. Lux and A. M. Brown, *J. Gen. Physiol.* **83**, 727 (1984)] in which idealized events of any given amplitude could be compiled into separate files. Nonlinear least squares and maximum likelihood criteria were used to estimate parameters for Gaussian distributions of amplitude frequency histograms.

10. For electrophysiological assays, fractions of GTP γ S-activated $r(+9)\alpha_s$ -S1 eluting from the DEAE column with highest *cyc*⁻ reconstituting activity (7) were pooled, mixed with [^3S]GTP γ S (100,000 cpm/ μl), and concentrated between 10- and 15-fold in Amicon Centricon concentrators. The concentrates were diluted in 10 mM $MgCl_2$, 1 mM EDTA, 20 mM β -mercaptoethanol, 10 mM sodium Hepes (pH 8.0), and 30% (v/v) ethylene glycol (buffer A) lacking the ethylene glycol and reconcentrated five times, after which time the sample was again made 30% in ethylene glycol. In this way the added GTP γ S was reduced to no more than 3 nM. Activation of Ca^{2+} channels by GTP γ S was not detected at concentrations below 20 nM. Aliquots were analyzed for reconstitution of GTP γ S-stimulated adenylyl cyclase activity in *cyc*⁻ membranes by using purified hRBC G_s^* as standard and, for mass of recombinant α subunits by SDS-polyacrylamide gel electrophoresis (PAGE), Coomassie blue staining and densitometric scanning. Varying amounts of bovine serum albumin (BSA) were coelectrophoresed to allow for quantification of the recombinant subunits in each lane. Typically, concentrated pools of GTP γ S-activated $r(+9)\alpha_s$ -S1 or $r(+9)\alpha_s$ -L1 contained 200 to 300 nM of recombinant protein ($M_r = 42,000$) and a *cyc*⁻ reconstituting equivalent to 2 to 4 nM hRBC G_s^* ; pools of GTP γ S-activated $r(+9)\alpha_s$ -3 preparations contained between 1000 and 2000 nM of the recombinant protein ($M_r = 40,000$). GTP α S-activated $r(+9)\alpha$ preparations were kept in buffer A at -70°C until used. GTP γ S-activated α_s -S2 and α_s -L1 were prepared as in (3) and kept at 0° to 4°C in 20 mM tris, 1 mM EDTA, 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, and 100 mM NaCl until used. Protein samples were then diluted with solution A in the cold and added to the 200- μl cis (or trans) chamber in 10- μl aliquots.
11. Bovine cardiac sarcolemmal vesicles were prepared from ventricle muscle [S. Slaughter, J. L. Sutko, J. P. Reeves, *J. Biol. Chem.* **258**, 3183 (1983)] and stored at -80°C . Vesicles were added to the cis chamber to a final concentration of 5 to 10 μg of protein per milliliter. Unlike skeletal muscle T-tubule vesicles, these vesicles were right-side out (1) and the solution in the trans chamber represented the intracellular solution. Cardiac Ca^{2+} channels are not continuously active at depolarized potentials and were activated by depolarization to -10 , 0, and $+10$ mV for 1 to 1.5 s from holding potentials of -70 or -40 mV at 2 Hz (1).
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Stretch-Inactivated Ion Channels Coexist with Stretch-Activated Ion Channels

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Stretch-activated ion channels of animal, plant, bacterial, and fungal cells are implicated in mechanotransduction and osmoregulation. A new class of channel has now been described that is stretch-inactivated. These channels occur in neurons, where they coexist with stretch-activated channels. Both channels are potassium selective. The differing stretch sensitivities of the two channels minimize potassium conductance over an intermediate range of tension, with the consequence that, over this same range, voltage-gated calcium channels are most readily opened. Thus, by setting the relation between membrane tension and transmembrane calcium fluxes, stretch-sensitive potassium channels may participate in the control of calcium-dependent motility in differentiating, regenerating, or migrating neurons.

STRETCH-ACTIVATED (SA) CHANNELS have been studied in diverse cell types (1-4). In snail neurons, under conditions used to study SA K⁺ channels (5, 6), some membrane patches were observed in which suction through the recording pipette not only activated the ubiquitous SA channels, but simultaneously inhibited a class of lower conductance, spontaneously active channels. In a single-channel recording where SA channel activity was abolished by quinidine, spontaneous currents (multiples of ~1 pA) were evident in the absence of external membrane tension and were abolished by suction (Fig. 1A). By contrast, more typical patches of membrane did not exhibit stretch-inactivated (SI) currents (7) but were nearly quiescent until stretch evoked SA channel activity (Fig. 1B). We suspect that even with the pipette tip tension nominally at zero, there was often sufficient residual suction (from capillarity) to inactivate the SI channels. This would explain why it was sometimes possible to stimulate activity by applying 3 to 5 mmHg of positive pressure; this activity subsided with further positive pressure, as expected for an SI channel. The occurrence of SI and SA (8) currents together (Fig. 1, C, D, and E) in a single patch from cell bodies or growth cones demonstrates that distinct populations of channels with reciprocal responses to mechanical tension can coexist within a

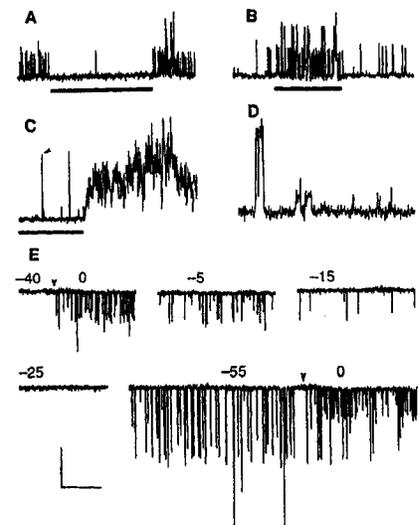
membrane area of several square micrometers.

To exclude the possibility that suction inhibits channel activity not because of increased membrane tension but because the

Fig. 1. Currents inhibited and activated by membrane stretch coexist. (A) Activity of SI channels in a cell body patch abolished by application of about -40 mmHg. NS plus 1 mM quinidine (18) in pipette; membrane potential, $V_m = +70$ mV. (B) SA channel activity stimulated by -40 mmHg. NS in pipette; $V_m = +70$ mV. Sustained responses in (A) and (B) were typical; note different amplitude calibration. (C) SA channel (arrowhead) and SI channel activity in the same patch, -70 mmHg, NS in pipette, $V_m = +70$ mV. (D) Higher resolution trace of SA channel and SI channel events from a growth cone, 0 mmHg, 50 mM K⁺ in pipette, patch $V_m = V_{rest} + 120$ mV (the resting potential, V_{rest} was unknown for growth cones). (E) SI and SA channels in a growth cone patch. Suction (in millimeters of mercury) is indicated adjacent to each segment of record; at the arrowheads, suction was abruptly released. Note that between about -20 and -50 mmHg, neither channel type was active. Fifty millimolar KCl in the pipette; patch held at V_{rest} . Calibration: (A) 1 pA, 2 s; (B) 4 pA, 2 s; (C) 2 pA, 1 s; (D) 2 pA, 40 ms; (E) 4 pA, 2 s. Traces (A), (B), (D), and (E) filtered at 1 kHz, (C) at 500 Hz. Solid bars below traces (here and in Fig. 3) indicate suction. *Lymnaea stagnalis* neurons were isolated from circumesophageal ganglia (45 min in 0.25% protease), plated on glass cover slips, and used for up to 4 days after dissociation. Cells were maintained in NS (50 mM NaCl, 1.6 mM KCl, 3.5 mM CaCl₂, 2 mM MgCl₂, 5 mM Hepes, pH 7.6) supplemented with 5 mM glucose, 50 IU of penicillin per milliliter, and 50 μg of streptomycin per milliliter. All recordings were made on cells bathed in this solution (exception noted in Fig. 3) at room temperature. Patch-clamp recordings of single-channel activity were made with standard cell-attached gigaseal techniques (19); pipettes were made from Corning 7052 glass. Suction, applied through a port in the microelectrode holder, was measured and controlled with a Bio-tek transducer. Cell body membrane potentials are calculated on the assumption of $V_{rest} = -50$ mV [from intracellular recordings, $V_{rest} = -51 \pm 1.9$ mV (SEM), $n = 32$].

channel-bearing membrane is drawn against the inner wall of the pipette, amplitude histograms were examined for the presence of attenuated events. Under conditions where suction sharply decreased the open probability (P_o) for SI channels, the distribution of single channel amplitudes was unchanged (Fig. 2). Calcium-related artifacts (for example, Ca²⁺ inactivation induced by inward leak of Ca²⁺) are ruled out by the observation of SI events in Ca²⁺-free conditions (Fig. 3). Even with such artifacts eliminated, do membranes in fact encounter tensions comparable to those applied through the patch pipette? Probably they do; for a semipermeable membrane, a mere 1 mosM equals 18 mmHg. Moreover, at the advancing tip of crawling cells, motility generates forces ranging from 10⁻⁸ to 10⁻³ dyne/μm (9), so that experimental tensions [on the order of 10⁻⁵ to 10⁻⁴ dyne/μm for -10 mmHg in a typical patch (1)] are not excessive.

For the SI channel, as for the SA channel (5), analysis of single-channel current-voltage relations indicates that K⁺ is the physiologically permeant species. With normal saline (NS) in the pipette (Fig. 3), the zero-current potential for the SI channel was near the resting potential and, as expected for a channel with negligible Na⁺ and intracellular anion permeability, the channel did not pass inward current. When Na⁺ was re-



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